Mammalian target of rapamycin: immunosuppressive drugs offer new insights into cell growth regulation

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Introduction

In this "enlightened" era of drug development, molecular targets are validated on the basis of their relevance to specific disease states, and screening assays are developed to identify small molecule- or peptide-derived modulators of the selected target's function. However, the more classical paradigm, in which the clinical application of new compounds frequently preceded detailed studies of their molecular mechanisms of action, has not been entirely abandoned. Relevant examples are the natural product immunosuppressive agents, cyclosporine A, FK506, and rapamycin. These drugs (cyclosporine A and FK506 in particular) had already made indelible marks on the clinical field of organ transplantation by the time that bench scientists had begun to unravel the molecular pharmacology underlying their effects on immune responses. Remarkably, the insights provided by basic investigations into the cellular mechanisms of action of the immunosuppressants have been as impressive as the results obtained with these drugs in the clinical arena. In each case, the availability of the immunosuppressant enabled investigators to uncover novel and largely unexpected pathways of intracellular signaling. Ongoing research using cyclosporine A, FK506, and rapamycin as pharmacologic probes continues to yield new information relevant to the clinical management of organ transplants, autoimmune diseases, inflammation, and even cancer.

The focus of this brief monograph will be on rapamycin, the latest member of the group to enter the clinic, and the compound whose intracellular target was most recently identified. Although we are a long way from a complete understanding of the mechanism of action of rapamycin, it is clear that this drug interferes with a cell growth-related signal transduction pathway that has been fundamentally conserved during eukaryotic evolution from yeast to man. As stated above, the recent progress toward the definition of this signaling pathway is due in large part to the availabil-

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ity of the highly specific inhibitor, rapamycin. The results to date have provided at least a partial molecular explanation for the decades-old observation that cell growth is most sensitive to inhibitors of protein synthesis during G_1 phase of the cell cycle [1]. From a therapeutic viewpoint, rapamycin may prove to be the first member of a very unique class of immunosuppressive and anticancer agents targeted against growth-regulatory proteins whose expression is controlled at the level of translation. For reasons that continue to elude cell biologists, the passage of immunocompetent, proinflammatory, and certain tumor cells through G_1 phase is particularly sensitive to disruption of the translational control pathway governed by the rapamycin target protein, mTOR.

Pharmacology of rapamycin

Rapamycin is a macrolide ester produced by a bacterial strain that was cultured from a soil sample collected during a search for novel antibiotics in the Easter Islands. The pharmacologic basis of rapamycin's cellular actions has been reviewed in detail [2], and will be summarized briefly in this monograph. The structures of rapamycin and the related compound, FK506, are shown in Figure 1. Rapamycin is a hydrophobic macrolide ester that binds to a highly conserved and ubiquitously expressed cytoplasmic receptor termed FK506-binding protein-12 (FKBP12). The resulting FKBP12•rapamycin complex acquires an activity not expressed by either component of the complex in isolation, i.e. the ability to bind to and inhibit the kinase activities of specific target proteins termed, appropriately enough, target of rapamycin, or TOR proteins. Several aspects of this pharmacologic mechanism of action are worthy of special mention. First, rapamycin shares a common receptor, FKBP12, with the structurally related immunosuppressive agent, FK506. Moreover, the binding of FK506 to FKBP12 also represents an activation step leading to the formation of a proximate enzyme inhibitor. However, the similarity stops here, in that the FKBP12•FK506 complex targets a completely different signaling molecule, the Ca^{2+}-regulated serine-threonine phosphatase, calcineurin. This phosphatase is neither recognized nor inhibited by the FKBP12•rapamycin complex, and the converse is true as well – TOR protein function is unaffected by treatment of cells with FK506. Indeed, the drugs may be considered as mutual antagonists, because concomitant exposure of cells to FK506 and rapamycin may set up a competition for a limiting amount of the FKBP12 receptor protein. From the drug development viewpoint, the mechanism of action of rapamycin represents a fascinating solution to the daunting task of designing a small molecule inhibitor bearing a high level of specificity for a large polypeptide target. The FKBP12 receptor not only positions rapamycin in the optimal orientation to interact with the TOR proteins, but also supplies structural determinants that contribute to the affinity and specificity of this interaction.

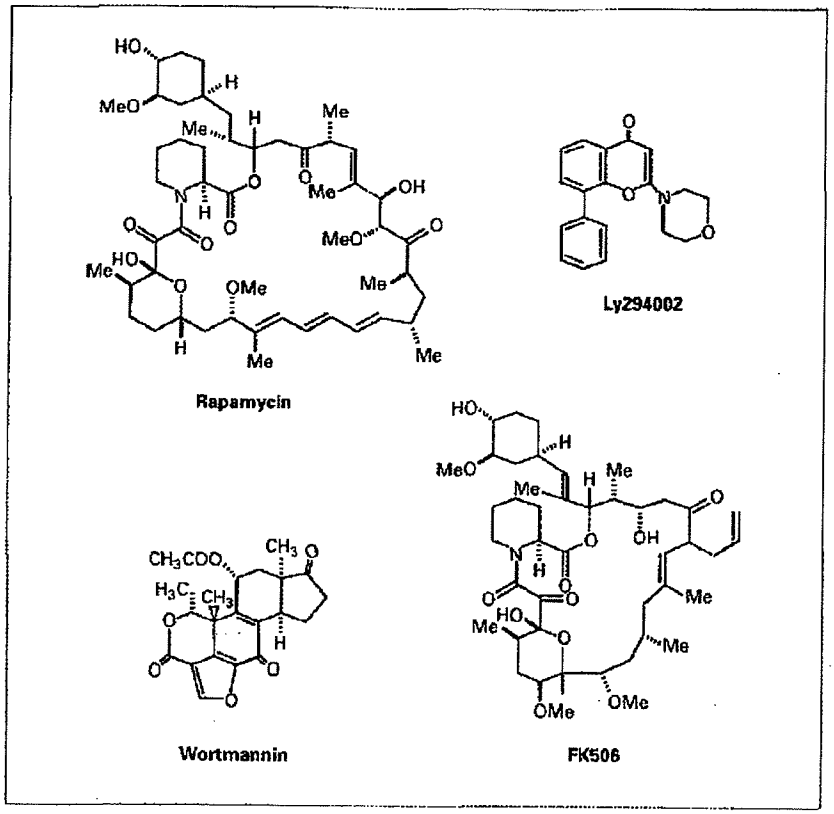


Figure 1
Structures of the macrolide immunosuppressants, FK506 and rapamycin, and the PI3K inhibitors, wortmannin and LY29002. Wortmannin binds irreversibly to and inhibits the catalytic activities of PI3Ks and many PIKKs, while the bioflavonoid derivative reversibly inhibits the catalytic activities of certain PI3Ks and PIKKs, including mTOR.

Identification of the rapamycin target protein

The strategy for the isolation of the rapamycin target protein was predicated on earlier work that led to the identification of calcineurin as the common ligand for the immunosuppressive complexes formed between FKBP12 and FK506, and cyclophilin A and cyclosporine A [3]. Mammalian tissue extracts were fractionated

to varying degrees, and then were passed over an affinity column containing FKBP12 loaded with rapamycin. Protein microsequence analysis led to the isolation of the full-length cDNA encoding the FKBP12•rapamycin-binding protein, which was named FRAP [4], RAFT1 [5], or mTOR [6] by the different laboratories. We termed this binding protein mammalian target of rapamycin (mTOR), in deference to the precedent nomenclature from yeast (see below).

The protein encoded by the mTOR cDNA was a complete surprise on several counts. First, the open reading frame encoded a very large polypeptide containing 2,549 amino acids, with a predicted molecular mass of 289 kilodaltons. Second, the only recognizable region of homology to other mammalian proteins resided near the carboxyl-terminus, which contained a stretch of approximately 400 amino acids that bore a distant but significant resemblance to the catalytic domains of phosphoinositide (PI) kinases, particularly those of PI 3-kinases (PI3Ks). Finally, the target protein, like the rapamycin receptor, FKBP12, was ubiquitously expressed in mammalian tissues and cells, with very high levels found in non-proliferating tissues, including brain and muscle. The latter results seemed in conflict with the observations that rapamycin did not behave like a broad-spectrum inhibitor of cell growth when administered to animals or humans. As stated above, a satisfactory explanation for the widely varying sensitivities of different tissue and cell types to rapamycin has not yet been proffered. What was recognized very quickly was that mechanistic studies of an immunosuppressive drug had uncovered a novel regulator of G_1 phase progression in mammalian cells. The molecular cloning of mTOR drew the attention of many investigators to a largely unappreciated area of signal transduction – the coupling pathway between growth factor receptor occupancy and the translational machinery in eukaryotic cells.

The deduced amino acid sequence of mTOR also told mammalian cell biologists that, as frequently has been the case in the signaling field, the yeast geneticists had "beaten them to the punch". In addition to its potent immunosuppressive activities, rapamycin is a powerful antifungal agent. Genetic screens for mutations that rendered the budding yeast, *Saccharomyces cerevesiae*, resistant to rapamycin yielded one known and two novel genes [7–9]. Mutations in the previously identified *FKP1* gene, which encodes the yeast FKBP12 ortholog, caused loss of rapamycin sensitivity. This resistant phenotype would be predicted on the basis of the pharmacology outlined above, as both FKBP12 and rapamycin are required for the cellular effects of the immunosuppressive drug. In addition, the screen revealed that mutations in either of two novel and highly related genes, *TOR1* and *TOR2*, permitted yeast cells to form colonies when plated onto a rapamycin-containing semisolid matrix. Remarkably, the sequences of the two yeast proteins exhibited greater than 40% overall identity to that of mTOR, and this sequence identity between the yeast and mammalian TORs rose to greater than 65% in the carboxyl-terminal region containing the PI3K-related catalytic domain. Clearly, TOR1p and TOR2p were orthologs of the subsequently identified mTOR polypeptide. When considered in

light of the observation that rapamycin treatment arrests the growth of yeast cells in G_1 phase of the cell cycle, it became evident that the TOR proteins participated in a cell-cycle regulatory pathway that had been fundamentally conserved during the evolution of eukaryotic cells.

Additional genetic studies in *S. cerevesiae* demonstrated that the *TOR2* gene was essential for viability, while the *TOR1* gene was nonessential, although loss of *TOR1* caused yeast cells to grow more slowly in nutritious medium [10, 11]. Interestingly, a double disruption of the *TOR1* and *TOR2* genes resulted in G_1-phase growth arrest and gradual loss of viability – a phenotype that strongly resembled the response of wild-type yeast cells to rapamycin exposure. These results further solidified the argument that rapamycin exerted its cellular effects by inducing loss of a critical signaling function(s) of TOR1p and TOR2p in G_1-phase yeast cells.

Biochemical insights into the mechanicsm of action of rapamycin

The identification of mutant TOR1p and TOR2p polypeptides that led to rapamycin resistance in yeast provided valuable clues regarding the nature of the interaction of the TOR proteins with the FKBP12•rapamycin complex. The "hot spot" for the generation of biologically active but drug-resistant TOR proteins was a conserved serine residue in TOR1p and TOR2p that, when substituted by a more bulky amino acid (e.g. arginine or isoleucine), rendered the host cells resistant to rapamycin [7, 12–14]. We now know that this critical serine residue, which is also found in the mammalian ortholog, mTOR, is nested within a ~100 amino acid stretch of amino acids termed the FKBP12•rapamycin binding (FRB) domain [13, 14] (see Fig. 2). Interestingly, the FRB domain is located immediately upstream of the catalytic domain, and has no identifiable role in catalysis. Nonetheless, the interaction of this domain with the FKBP12•rapamycin complex strongly inhibits the kinase activity of mTOR. Although the mechanism remains unclear, a speculative proposal is that the bulky immunophilin•drug complex poses a steric hindrance to the presentation of protein substrates to the catalytic domain.

The findings described above highlight two pharmacological features that have greatly facilitated research concerning the signaling functions of mTOR in mammalian cells. First, rapamycin is an exquisitely specific inhibitor of mTOR function in intact cells; hence, any alteration in a cellular response induced by rapamycin strongly implicates a role for mTOR in the pathway leading to the response. Second, the availability of rapamycin-resistant, but otherwise fully functional, mTOR mutants permits detailed structure-function studies of this kinase in otherwise wild-type cells. Such mutants are readily generated by substitution of Ser^{2035} in the FRB domain of mTOR with a more bulky substituent, such as Ile. The general strategy is then to introduce the rapamycin-resistant mTOR mutant into the appropriate cellular host, and then to treat these cells with rapamycin. The drug is assumed to

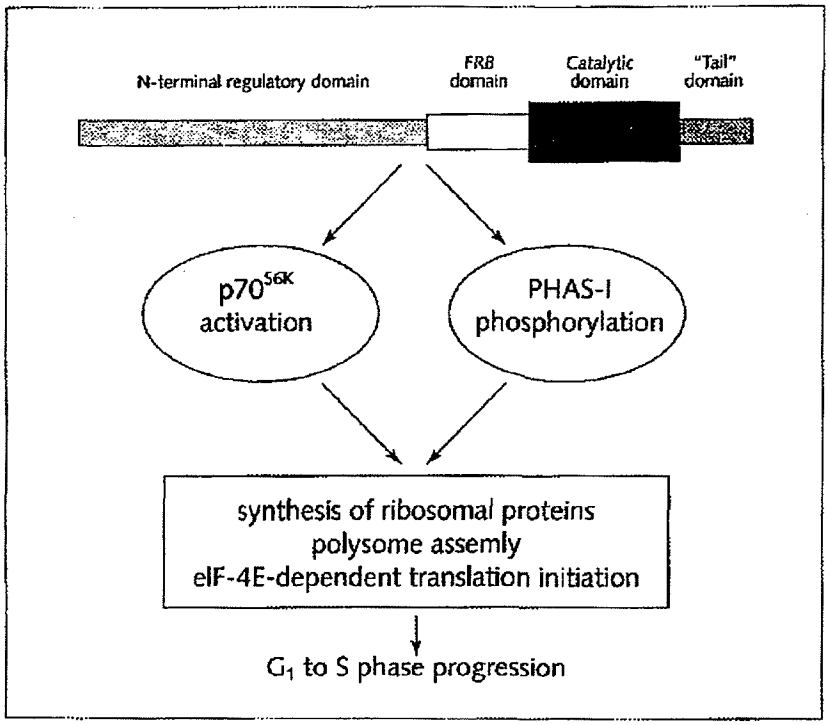
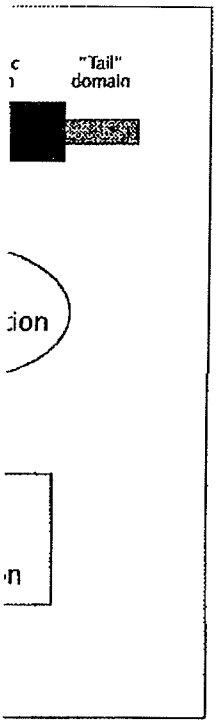


Figure 2

Structure and rapamycin-sensitive signaling functions of mTOR. The conceptual translation product of the mTOR cDNA is a 2,549 amino acid polypeptide containing a carboxyl-terminal region similar to the catalytic domains of PI3Ks. The function of the very extended N-terminal region (~ 1900 amino acids) is unclear, but this domain may play roles in protein-protein interactions and/or the subcellular localization of mTOR. The FRB domain comprises approximately 100 amino acids, and represents the binding site for the inhibitory FKBP12-rapamycin complex. The function of the "tail" domain, which contains approximately 30 amino acids, is also unclear, but deletion experiments indicate that this domain is essential for the protein kinase activity of mTOR.

The kinase activity of mTOR is required for the phosphorylation of $p70^{S6K}$ and PHAS-I in mitogen-stimulated cells. Activation of $p70^{S6K}$ facilitates the overall process of translation initiation, and specifically increases the synthesis of components of the protein synthesis machinery itself. The phosphorylation of PHAS-I leads to an increase in eIF-4E-dependent translation initiation, and may specifically augment the production of proteins required for G_1 to S phase progression.



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effect a functional "knockout" of the rapamycin-sensitive functions of the endogenous mTOR proteins in these cells, thereby allowing one to focus on the signaling capabilities of the ectopically-expressed mTOR mutant. Although this approach has limitations, it has been instrumental in efforts to define the signaling functions of mTOR in mammalian cells.

A family of PI3K-related kinases

The months that followed the cloning of mTOR were marked by a flurry of activity leading to the identification of a large and still-growing family of signaling proteins that contain the PI3K-like catalytic domain. This novel family of proteins, which we term PI3K-related kinases (PIKKs), has been the subject of several reviews [15-17]. The PIKK family currently contains four mammalian proteins: mTOR, ataxia telangiectasia-mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK). A mammalian cDNA encoding a Myc-interacting protein, termed TRRAP, may be the newest addition to the PIKK family [18]. However, the deduced sequence suggests that the region of similarity to the PI3K catalytic domain may not possess phosphotransferase activity. In contrast to the TOR proteins, the remaining members of the PIKK family appear not to be involved in the transmission of mitogenic signals from the cell surface. Rather, this subgroup of PIKKs, which includes ATM, ATR, and DNA-PK, participate in genome surveillance and maintenance by functioning as components of cell-cycle checkpoints and the DNA repair machinery. This subgroup has attracted considerable attention from cancer researchers, because a hallmark characteristic of cancer cells is genomic instability, a phenotype that can be traced to the breakdown of one or more cell-cycle checkpoints. The importance of cell-cycle checkpoints during normal development is underscored in dramatic fashion by the catastrophic consequences of the loss of ATM function in humans. AT patients exhibit chromosome instability leading to neurodegeneration, immunodeficiency, elevated cancer susceptibility, and hypersensitivity to radiation and other DNA-damaging agents. It is particularly noteworthy, that, at this early stage of our understanding, the PIKK family already holds a novel target (mTOR) for a clinically relevant immunosuppressant, and a checkpoint kinase (ATM) whose loss explains the complex phenotype of a long-studied human disease.

Effects of rapamycin on mammalian cell growth

One notable difference between yeast and mammalian cells is that the latter cells display widely varying sensitivities to the growth-inhibitory effect of rapamycin, depending on the cell lineage, as well as the growth factor milieu in which the

response to the drug is being evaluated. Although the actual determinants of rapamycin sensitivity or resistance have not been defined, recent insights into the mTOR-dependent signaling pathway may eventually yield the answer to this crucial question (see below). A prototypical target cell for rapamycin is the activated T lymphocyte, which undergoes G_1 to S phase progression in response to IL-2 or T-cell growth-promoting cytokines. IL-2-stimulated T cells accumulate in mid/late G_1 phase of the cell cycle in the presence of ≤ 10 nM rapamycin [19, 20]. The growth-arrested T cells are characterized by the presence of fully assembled but catalytically inactive cyclin E-Cdk2 complexes, the maintenance of the under-phosphorylated, and hence growth-suppressive form of the retinoblastoma protein (pRb), in spite of continuous exposure to IL-2. Rapamycin treatment seems to allow T cells to progress up to the so-called G_1 restriction point, but does not permit passage through this important checkpoint. Restriction point traverse, which depends on the hyperphosphorylation, and inactivation of pRb marks the transition from growth factor-dependent cell-cycle progression to an intrinsic commitment of the cell-cycle machinery to execute S, G2, and M phases [1]. Thus, the rapamycin target, mTOR, must mediate the delivery of signals required for the progression of G_1-phase T cells through the restriction point.

Role of mTOR in translational control

The search for more proximal biochemical responses to rapamycin treatment led to the identification of two proteins whose phosphorylation state is regulated by mTOR. The first target protein is p70 S6 kinase ($p70^{S6K}$), a serine-threonine kinase that is activated in response to a broad range of mitogenic stimuli. Rapamycin blocks both the phosphorylation and activation of $p70^{S6K}$ in all mammalian cell types examined to date. The second endpoint for the mTOR-dependent phosphorylation pathway is the translational-repressor protein, PHAS-I (also termed 4E-BP1) [21]. As mentioned above, it is striking that both $p70^{S6K}$ and PHAS-I participate in the regulation of protein synthesis in cells stimulated with mitogens or certain hormones, including insulin.

The regulation of $p70^{S6K}$ by upstream protein kinases is exceedingly complex, and the reader is referred to specialized reviews for details concerning this topic [22, 23]. However, it is clear that treatment with rapamycin quickly and efficiently inhibits the *de novo* phosphorylation of $p70^{S6K}$ induced by hormonal stimuli, as well as the phosphorylation of previously activated $p70^{S6K}$. The predicted epistatic relationship between mTOR and $p70^{S6K}$ was confirmed in cell transfection experiments, which demonstrated that introduction of a rapamycin-resistant mTOR mutant into Jurkat T cells rendered $p70^{S6K}$ activation correspondingly resistant to rapamycin [24]. The only documented physiologic substrate for $p70^{S6K}$ is the 40S ribosomal protein S6, although recent data suggest that this protein kinase also

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phosphorylates two translation initiation factors, eIF-4G and eIF-4B [25]. The overall effect of these phosphorylation events is to increase the capacity of the protein synthetic machinery to translate mRNA templates. This modulation of translational capacity is logical, when one considers that successful passage through G_1 requires a substantial increase in cell mass, if the mitotic cycle is destined to give rise to two normally sized daughter cells.

Regulation of translation initiation by mTOR

The rate-*limiting step in* the translation of most eukaryotic mRNAs is initiation, a process that includes the binding of the 43S ribosomal preinitiation complex to the 5'-terminus of the mRNA, and the 5'→3' translocation of this complex as it scans the 5'-UTR for an AUG initiation codon [25, 26]. Both the ribosome binding and scanning steps are facilitated by the eukaryotic initiation factor (eIF)-4F complex, which itself binds to the cap structure (m^7GpppN, where N is any nucleotide) found at the extreme 5'-terminus of nearly all eukaryotic mRNAs. The eIF-4F complex contains eIF-4G, a large scaffolding protein, eIF-4A, an ATP-dependent RNA helicase (when partnered with an additional initiation factor, eIF-4B), and eIF-4E, the mRNA cap-binding subunit. An exciting realization over the past several years is that rates of translation initiation are controlled by extracellular stimuli, including growth factors, cytokines, and insulin. Moreover, this regulatory mechanism is highly discriminate: in mitogen-stimulated cells, translation of some mRNAs increases dramatically (> 30-fold) while the overall increase in protein synthesis is quite modest (1.5–2-fold).

The major determinants of eIF-4F dependence reside within the 5'-UTRs of translatable mRNAs. In quiescent cells, mRNAs bearing 5'-UTRs with extensive secondary structure and/or multiple upstream open reading frames tend to be translated very inefficiently due to impaired initiation. Interestingly, a number of growth-regulatory proteins (e.g. c-Myc, cyclin D_1 and ornithine decarboxylase) are encoded by mRNAs that contain such structural complexity in their 5'-UTRs. Mitogenic stimuli increase the translational efficiencies of these mRNAs by stimulating eIF-4F binding and function, often through the phosphorylation of specific components of this complex. Collectively, this "activated" eIF-4F increases both ribosome binding to the mRNA, and simplifies the structure of the 5'-UTR *via* the RNA-unwinding activity of eIF-4A, acting in concert with eIF-4B. The positive regulatory effect of mitogens on eIF-4F function therefore provides a mechanism by which the expression of certain growth-related genes can be controlled at the translational level.

The cap-binding eIF-4E subunit is a target for multiple intracellular signaling pathways, including the pathway governed by mTOR. The interaction of eIF-4E with the remaining components of the eIF-4F complex is competitively inhibited by the formation of complexes with 4E-binding proteins (4E-BPs) [21, 27]. These eIF-

4E interactors are also termed PHAS (phosphorylated heat and acid stable) proteins, and, for historical reasons, we will use the latter terminology for the remainder of this discussion. The most well-studied member of this family of eIF-4E inhibitors is PHAS-I. In quiescent cells, PHAS-I is not phosphorylated, and is tightly bound to eIF-4E. Under these conditions, eIF-4F function, and therefore translation initiation, is repressed. Exposure of these cells to growth factors or insulin results in the rapid phosphorylation of PHAS-I at 5 serine or threonine residues, and a consequent decrease in the binding affinity of PHAS-I for eIF-4E. Thus, the multi-site phosphorylation of PHAS-I removes a significant obstacle to eIF-4E-dependent translation initiation, and facilitates the synthesis of proteins needed for G_1-phase progression.

The protein kinase(s) responsible for the phosphorylation of PHAS-I quickly became a topic of considerable interest. A seminal observation was that this response was blocked by growth-inhibitory concentrations of rapamycin, which strongly hinted that mTOR served as an upstream kinase in the PHAS-I phosphorylation pathway [28–30]. In a somewhat unexpected turn of events, it was recently shown that mTOR itself phosphorylates PHAS-I, at least under *in vitro* kinase assay conditions [31]. The physiologic relevance of these findings is supported by the findings that the five sites phosphorylated by mTOR *in vitro* are identical to the sites of PHAS-I phosphorylation during insulin stimulation of intact cells [32, 33]. The same serine and threonine residues are rapidly dephosphorylated upon addition of rapamycin to these cells. Finally, the *in vitro* phosphorylation of PHAS-I by mTOR effectively inhibits the binding of PHAS-I to eIF-4E. Collectively, these findings argue that mTOR may be directly responsible for the phosphorylation of PHAS-I and subsequent activation of eIF-4E induced by insulin and other mitogenic factors.

Regulation of mTOR activity by hormonal stimuli

An important area for ongoing studies concerns the pathways through which mTOR is regulated in response to extracellular stimuli. Signals emanating from activated PI3K have been implicated in the stimulation of protein synthesis for some time. More recent evidence suggests that both PI3K and its downstream serine-threonine kinase, AKT, participate in mTOR activation by insulin and other polypeptide hormones [34, 35]. Interestingly, the carboxyl-terminal region of mTOR contains at least two consensus sites for phosphorylation by AKT, and indirect evidence suggests that these sites are, in fact, phosphorylated in an AKT-dependent fashion in intact cells [35]. These findings also raise some cautionary points concerning the extensive laboratory application of wortmannin, an irreversible inhibitor of PI3K, as a signal transduction inhibitor in mammalian cells. Treatment of cells with wortmannin will interfere with at least two components of the cytokine receptor-linked pathway leading to translation initiation: the lipid kinase activity of the p85-p110

form of PI3K and, as reported recently, the protein kinase activity of mTOR [31]. Wortmannin irreversibly inhibits the PHAS-I phosphorylating activity of mTOR with an IC_{50} of 250 nM [36]. Although the potency of wortmannin as an mTOR kinase inhibitor is approximately 100-fold lower than that observed with p85-p110 as the target (IC_{50}, 3 nM), the emergence of mTOR and other members of the PIKK family [37] as wortmannin-sensitive kinases complicates the interpretation of results based on the use of this broad spectrum inhibitor of kinases containing the PI3K-related catalytic domain.

Summary and perspective

Rapamycin is a potent immunosuppressive drug that seems destined to find important applications in the transplantation clinic, and in the treatment of autoimmune disease and, possibly, certain types of cancer. Studies of the mechanism of action of rapamycin indicate that this drug blocks the growth of lymphoid and other cell types by suppressing the protein kinase activity of mTOR. The rapamycin target protein, mTOR, functions in a highly conserved signaling pathway leading to the activation of the translational enhancer, $p70^{S6K}$ and the functional inactivation of the translational repressor, PHAS-I. The mTOR signaling pathway seems to be required for the translation of certain mRNAs whose protein products carry out functions permissive for the passage of G_1-phase cells through the restriction point. The discovery that mTOR is a serine-threonine protein kinase should facilitate the further development of new drugs that, like rapamycin, interfere in a relatively subtle fashion with the biochemical pathway that links cytokine receptor occupancy to the synthesis of proteins involved in the control of cell growth.

From a broader perspective, the use of rapamycin as a pharmacologic probe opened an avenue of investigation that led to the identification of a completely novel family of signaling proteins, the PIKKs. Ongoing studies of the PIKKs will provide novel insights into the mechanisms whereby normal cells regulate their growth and maintain the integrity of their genomes. The information already available strongly suggests that PIKK dysfunction will lead to defects in the development of the nervous and immune systems, and will favor the development of cancer in humans. At the same time, however, members of the PIKK family have considerable potential as targets for the development of novel anticancer and immunosuppressive agents.

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Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation

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The immunosuppressant drug rapamycin blocks progression of the cell cycle at the G_1 phase in mammalian cells and yeast. Here we show that rapamycin inhibits cap-dependent, but not cap-independent, translation in NIH 3T3 cells. Cap-dependent translation is also specifically reduced in extracts from rapamycin-treated cells, as determined by *in vitro* translation experiments. This inhibition is causally related to the dephosphorylation and consequent activation of 4E-BP1, a protein recently identified as a repressor of the cap-binding protein, eIF-4E, function. These effects of rapamycin are specific as FK506, a structural analogue of rapamycin, had no effect on either cap-dependent translation or 4E-BP1 phosphorylation. The rapamycin–FK506 binding protein complex is the effector of the inhibition of 4E-BP1 phosphorylation as excess of FK506 over rapamycin reversed the rapamycin-mediated inhibition of 4E-BP1 phosphorylation. Thus, inactivation of eIF-4E is, at least in part, responsible for inhibition of cap-dependent translation in rapamycin-treated cells. Furthermore, these results suggest that 4E-BP1 phosphorylation is mediated by the FRAP/TOR signalling pathway.

Keywords: 4E-BP1/cap-dependent translation/rapamycin

Introduction

Rapamycin forms a complex with the immunophilin FKBP (FK506 binding protein). This complex binds to a family of kinases named FKBP-rapamycin-associated protein (FRAP) in humans, rapamycin and FKBP12 target (RAFT) in rats and target of rapamycin (TOR) in yeast (Heitman *et al.*, 1991; Kunz *et al.*, 1993; Brown *et al.*, 1994; Sabatini *et al.*, 1994; Stan *et al.*, 1994; Sabers *et al.*, 1995). In yeast, rapamycin is a potent inhibitor of translation (>90%) (Barbet *et al.*, 1996). However, in mammalian cells, rapamycin causes partial inhibition of translation between 15 and 40% in different cell lines (Jefferies *et al.*, 1994; Terada *et al.*, 1994). Rapamycin prevents the mitogenic activation of p70 S6 kinase, which phosphorylates the ribosomal protein S6 in mammalian cells (Chung *et al.*, 1992; Price *et al.*, 1992; Thomas, 1993). In contrast, in yeast, phosphorylation of the S6 homologue is not required for translation and growth (Johnson and Warner, 1987).

Translation rates are modulated in response to growth factors, hormones and mitogens. Most of this control is at the level of translation initiation. Translation initiation functions to position the ribosome at the AUG initiation codon. Cellular mRNAs contain a cap structure [$m^7G(5')ppp(5')N$; where N is any nucleotide] at their 5′ terminus (Shatkin, 1985). Recently, a novel repressor of cap-mediated translation, termed 4E-BP1 (eIF-4E binding protein-1) or PHAS-I, was characterized (Lin *et al.*, 1994; Pause *et al.*, 1994). 4E-BP1 is a heat- and acid-stable protein whose activity is regulated by phosphorylation (Hu *et al.*, 1994; Lin *et al.*, 1994; Pause *et al.*, 1994). 4E-BP1 interacts with the mRNA 5′ cap-binding protein, eIF-4E. Interaction of 4E-BP1 with eIF-4E results in the specific inhibition of cap-dependent translation, both *in vitro* and *in vivo* (Pause *et al.*, 1994). Phosphorylation of 4E-BP1 in response to treatment of cells with insulin or growth factors, decreases 4E-BP1 affinity for eIF-4E and thus relieves translational inhibition (Lin *et al.*, 1994; Pause *et al.*, 1994).

Translation of most eukaryotic mRNAs is cap-dependent (Jackson *et al.*, 1990). However, some viral and cellular mRNAs contain an internal ribosomal entry site (IRES) which promotes translation by a cap-independent mechanism (Jackson *et al.*, 1990). We investigated the possibility that rapamycin represses specifically cap-dependent translation in mammalian cells, as was suggested for yeast (Barbet *et al.*, 1996), and we examined the role of 4E-BP1 in this process.

Results

Rapamycin reduces cap-dependent translation in vivo

Rapamycin reduced protein synthesis (~2-fold after 20 h) in NIH 3T3 cells as determined by metabolic labelling of cells with [^{35}S]methionine (Figure 1). We investigated the possibility that rapamycin represses specifically cap-dependent translation in mammalian cells, as was suggested for yeast (Barbet *et al.*, 1996). To this end, bicistronic mRNAs containing the IRES from encephalomyocarditis virus (EMCV) (Figure 2A) were expressed in NIH 3T3 cells. The translation of chloramphenicol acetyltransferase (CAT) is cap-dependent, while the translation of luciferase (LUC) directed by the EMCV IRES is cap-independent. Rapamycin caused a dose-dependent inhibition of the cap-dependent translation of CAT, whereas the cap-independent translation of LUC was not diminished (Figure 2A). Cap-dependent translation decreased maximally by 42% with 20 ng/ml of rapamycin.

This effect is specific to rapamycin, as FK506, a structural analogue of rapamycin which binds to the immunophilin FKBP, but elicits a different biological response (Schreiber, 1991), had no effect on either cap-dependent or cap-independent translation (data not shown). To substantiate these results, a different IRES element was used. In addition, CAT and LUC were reversed to exclude the possibility that the results reflect different protein stabilities of LUC and CAT. The translation of LUC from this construct (pGEMLUC/pol/CAT; Figure 2B) is cap-dependent, while the translation of CAT directed by the poliovirus IRES is cap-independent. Following transfection in NIH 3T3 cells, rapamycin (at 20 ng/ml) decreased cap-dependent translation of LUC by 47% without affecting the translation of CAT (Figure 2B). The rapamycin dose-dependent reduction in translation in these experiments correlated well with the growth inhibition of the cells (Figure 2C). Slight inhibition of growth (~20% after 2 days) was observed with 0.2 ng/ml of rapamycin, whereas maximal inhibition (~40% after 2 days) was obtained with 2 ng/ml. Thus, reduction in protein synthesis following rapamycin treatment results, at least in part, from a general inhibition of cap-dependent translation in NIH 3T3 cells.

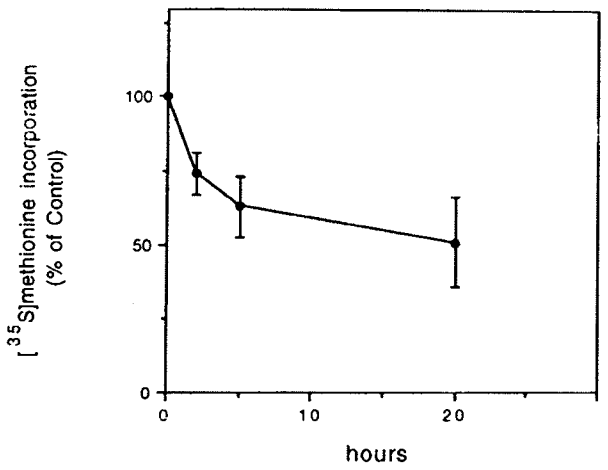

Fig. 1. Inhibition of protein synthesis by rapamycin. NIH 3T3 cells were serum-starved for 48 h and preincubated for 1 h in methionine-free medium. Rapamycin (10 mg/ml in ethanol) was diluted 1:100 in medium and added to the cells to a final concentration of 20 ng/ml together with [^{35}S]methionine (100 μCi) and serum (10%). Cells were harvested at different time periods and radioactivity incorporated into TCA-precipitable material was measured. The effect of rapamycin is expressed as percent of the control. The experiment was carried out three times and the error bars indicate the standard deviation from the mean.

In vitro translation in rapamycin-treated extracts

To demonstrate further that the rapamycin-mediated inhibition of translation is causally related to the inhibition of the cap function, we performed *in vitro* translation experiments. Because extracts from NIH 3T3 cells are inactive in translation, we used extracts from Krebs-2 mouse carcinoma cells. Capped and uncapped bi-cistronic mRNAs (pGEMCAT/EMC/LUC; Figure 2A) were translated in extracts from mock- and rapamycin-treated cells (Figure 3). In experiments performed with capped mRNAs,

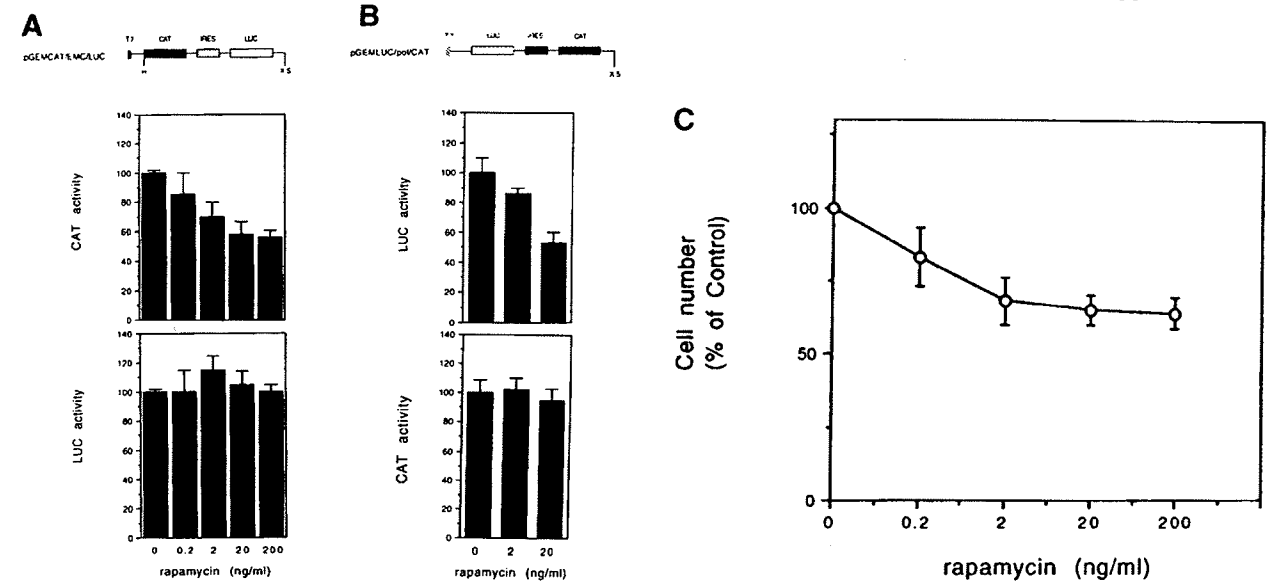

Fig. 2. Effect of rapamycin on translation. (**A**) pGEMCAT/EMC/LUC (Pause *et al.*, 1994) generates bicistronic mRNAs containing the IRES derived from the 5′ untranslated region of EMCV. The indicated restriction enzyme sites are as follows: H, *Hin*dIII; S, *Sac*I; X, *Xho*I. The coding sequence is under the control of the T7 promoter. NIH 3T3 cells were infected for 1 h with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase, followed by transfection with pGEMCAT/EMC/LUC (2 μg) using lipofectin (15 μl). After 4 h, rapamycin (0.2–200 ng/ml) or FK506 (20 ng/ml) and serum (10%) were added. Cell extracts were prepared 20 h post-transfection and analysed for CAT expression and LUC activity. The level of expression of CAT and LUC in control cells was set as 100%. The experiment was carried out four times and the error bars indicate the standard deviation from the mean. (**B**) pGEMLUC/pol/CAT (Pause *et al.*, 1994) generates bicistronic mRNAs containing the IRES derived from the 5′ untranslated region of poliovirus. The indicated restriction enzyme sites are as follows: S, *Sac*I; X, *Xho*I. The coding sequence is under the control of the T7 promoter. The experiment was carried out in duplicate and the error bars indicate the standard deviation from the mean. The level of expression of CAT and LUC in control cells was set as 100%. (**C**) NIH 3T3 cells were cultured to confluence. Cells were plated at a density of 10^6 cells per dish in medium containing serum (10%) and rapamycin (0–200 ng/ml). Cells were counted after 48 h. The experiment was performed in triplicate and the effect of rapamycin is expressed as percent of the control. The error bars indicate the standard deviation from the mean.

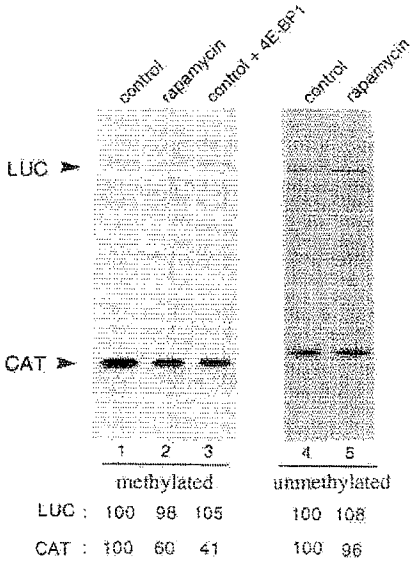

Fig. 3. Extracts prepared from rapamycin-treated cells mimic the *in vivo* reduction in cap-dependent translation. Plasmid pGEMCAT/EMC/LUC was linearized with *Xho*I and *in vitro*-transcribed with T7 RNA polymerase (Promega) for 2 h at 37°C. m^7GpppG or GpppG were included into the transcription mixtures in 10-fold molar excess relative to GTP to synthesize capped methylated or capped unmethylated mRNAs, respectively (Pelletier and Sonenberg, 1985). Krebs-2 mouse carcinoma cells were grown in suspension in S-MEM at 37°C for 2 h in the presence or absence of rapamycin (20 ng/ml). S_{30} translation extracts were prepared essentially as described (Svitkin and Agol, 1978; Svitkin *et al.*, 1984). Samples (12.5 µl) were incubated with [^{35}S]methionine for 90 min at 30°C in the presence of 0.15 µg capped methylated (lanes 1–3) or capped unmethylated (lanes 4 and 5) CAT/EMC/LUC mRNAs. In lane 3, translation was performed in the presence of 0.75 µg 4E-BP1. Following translation, reaction mixtures were supplemented with 2.5 µl buffer containing 6 mg/ml RNAse A and 60 mM EDTA and incubated at 30°C for an additional 5 min. Translation products were resolved by SDS–12% PAGE. The gels were processed for fluorography using En^{3}Hance. Quantification of the radioactivity incorporated into CAT and LUC proteins was performed using BAS-2000 PhosphorImager (Fuji Corp.) and expressed as percent of the control. The experiment was carried out four times, with <10% variation between the experiments.

translation of CAT is reduced by 40% in extracts from rapamycin-treated cells, whereas translation of LUC is unchanged (Figure 3, compare lanes 1 and 2). This effect of rapamycin is similar in its specificity (i.e. cap-dependent versus cap-independent) to the inhibition observed after addition of 4E-BP1 to control extracts (Figure 3, lane 3). In contrast, in experiments performed with an uncapped mRNA, no effect was observed on the translation of either CAT or LUC in rapamycin-treated extracts (Figure 3, lanes 4 and 5). These results clearly demonstrate that rapamycin inhibits *in vitro*, as it does *in vivo*, specifically cap-dependent, but not cap-independent translation.

Rapamycin inhibits phosphorylation of 4E-BP1

To study the mechanism by which rapamycin inhibits cap-dependent translation, the effect of rapamycin on 4E-BP1 was examined. Two isoforms of 4E-BP1 (indicated by the two upper arrows; Figure 4A, lane 1) were detected following immunoblotting of extracts from serum-starved NIH 3T3 cells. Serum stimulation of cells resulted in an increase in the amount of the slowest migrating electrophoretic isoform of 4E-BP1, most probably as a result of phosphorylation (Figure 4A, compare lanes 1 and 2). Strikingly, the two 4E-BP1 isoforms disappeared and a new faster migrating species (indicated by the lower arrow; Figure 4A, lane 3) became apparent in response to rapamycin. That the mobility shift is due to a decrease in 4E-BP1 phosphorylation was confirmed by [^{32}P]orthophosphate labelling of the cells followed by immunoprecipitation with an anti-4E-BP1 antibody. Rapamycin treatment caused a 6- to 8-fold reduction of ^{32}P incorporation in 4E-BP1, as measured by phosphorimager analysis (Figure 4A, lanes 4 and 5). As no incorporation of radioactivity was detected in the faster migrating isoform, this isoform is likely to be the dephosphorylated form of 4E-BP1. Rapamycin-mediated dephosphorylation of 4E-BP1 is not a consequence of a general inhibition of phosphorylation as similar patterns of labelled phosphoproteins between untreated and rapamycin-treated cells were observed in a 1-D SDS–PAGE (data not shown). Rapamycin also inhibited phosphorylation of 4E-BP1 in the Jurkat lymphoid cell line (data not shown), demonstrating that rapamycin blocks phosphorylation of 4E-BP1 in different cell types.

Figure 4B shows the kinetics of inhibition of phosphorylation of 4E-BP1 in response to rapamycin treatment. Whereas a small effect (~17%) was observed after 30 min of treatment, complete dephosphorylation was obtained after 1 h. The effect of rapamycin on 4E-BP1 phosphorylation is specific, as FK506 had no effect on 4E-BP1 phosphorylation in NIH 3T3 cells (Figure 4C, compare lanes 1 and 2). Also, cyclosporin A and FK506 had no effect on the phosphorylation of 4E-BP1 in Jurkat cells (data not shown). Because rapamycin and FK506 bind to a common receptor, FKBP, they are mutually antagonistic (Bierer *et al.*, 1990; Dumont *et al.*, 1990). To verify that a rapamycin–FKBP complex mediates the inhibition of 4E-BP1 phosphorylation, cells were treated with excess of FK506 over rapamycin. A 200-fold excess of FK506 over rapamycin partially reversed the rapamycin-mediated inhibition of 4E-BP1 phosphorylation and complete reversion was observed at a ratio 1000:1 (Figure 4C, lanes 3–6). Thus, the rapamycin–FKBP complex is the effector of the inhibition of 4E-BP1 phosphorylation. Furthermore, we found that an excess of FK506 also reversed the rapamycin-mediated inhibition of cap-dependent translation described in Figure 2A (data not shown).

Rapamycin-stimulated interaction between 4E-BP1 and eIF-4E

Rapamycin inhibition of 4E-BP1 phosphorylation is predicted to promote the interaction between the cap binding protein, eIF-4E and 4E-BP1. To test this prediction, eIF-4E fused to a peptide which contains the heart muscle kinase (HMK) phosphorylation site was labelled to a high specific activity and used as a probe in a 'far-Western' assay (Pause *et al.*, 1994). HMK–eIF-4E did not interact with the slowest migrating electrophoretic isoform (Figure 5, compare lanes 1 and 2) and interacted weakly with the middle isoform which is likely to have at least one phosphate group (see Figure 4A; Lin *et al.*, 1995), as phosphorylation of 4E-BP1 prevents its binding to eIF-4E (Pause *et al.*, 1994). This weak interaction was further diminished following the addition of serum, as a consequence of the phosphorylation of 4E-BP1 (Figure 5, compare lanes 2 and 3). However, a markedly enhanced

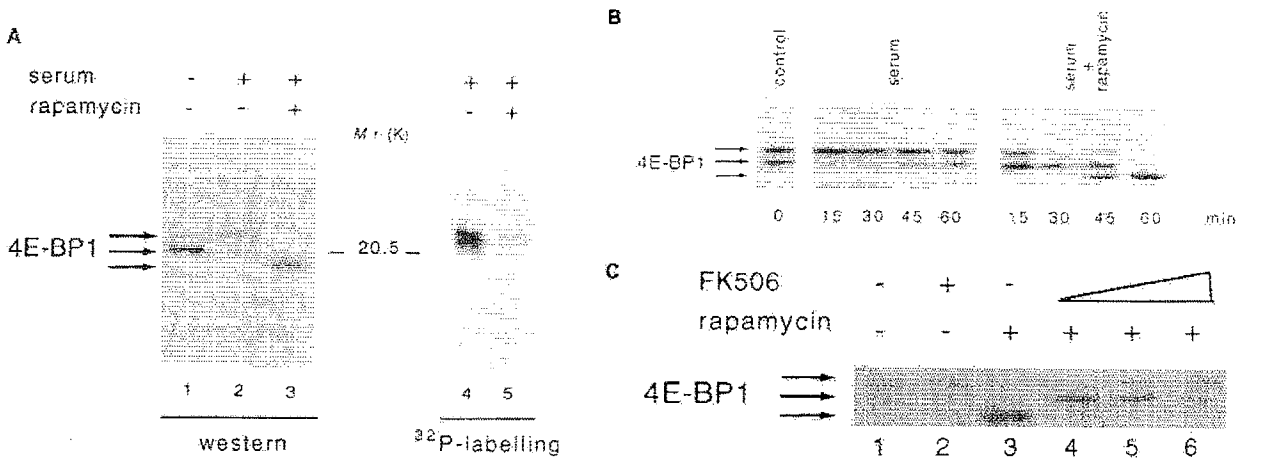

Fig. 4. Rapamycin blocks the phosphorylation of 4E-BP1. NIH 3T3 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Confluent plates were further cultured in Dulbecco's modified essential medium containing 0.5% FBS for 48 h prior to experiments. (**A**) For Western analysis, quiescent cells were treated for 3 h with serum in the presence or absence of rapamycin (20 ng/ml). Total protein extract was analysed by Western blotting using rabbit anti-4E-BP1 (1:1000) followed by [^{125}I]protein A (lanes 1–3). For ^{32}P-labelling and immunoprecipitation, quiescent NIH 3T3 cells were incubated in phosphate-free medium containing [^{32}P]orthophosphate (500 μCi per plate). After 1 h, serum with or without rapamycin was added for 3 h followed by cell lysis as described in Materials and methods. Incorporation of radioactivity into TCA-precipitated material was determined and samples containing 500 000 c.p.m. were incubated with rabbit anti-4E-BP1 antiserum (5 μl) and protein A–Sepharose (25 μl, Pharmacia) in Tris-buffered saline for 2 h at 4°C. Immunoprecipitated proteins were resolved on an SDS–15% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose membrane was exposed to an X-ray film (lanes 4 and 5). (**B**) Quiescent NIH 3T3 cells were treated with serum alone (10%) or with serum (10%) and rapamycin (20 ng/ml) as described in Figure 2A and harvested at various time periods. Proteins were analysed as described in Figure 2A. (**C**) Quiescent cells were treated for 1 h with serum (10%) in the presence or absence of FK506 (20 ng/ml) (lanes 1 and 2) or with serum (10%) and rapamycin (20 ng/ml) (lane 3) in the presence of excess FK506 (4–20 μg/ml) (lanes 4–6). Cells were harvested and proteins were analysed as described in Figure 2A.

interaction occurred with the fastest migrating dephosphorylated 4E-BP1 in response to rapamycin treatment (Figure 5, lane 4). An enhanced interaction between eIF-4E and 4E-BP1 was also observed in Jurkat cells following rapamycin treatment (data not shown). Thus, rapamycin increases the interaction of eIF-4E with 4E-BP1, resulting in the inhibition of cap-dependent translation.

Effect of rapamycin on eIF-4E phosphorylation

Previous reports have shown a correlation between the phosphorylation state of eIF-4E and translation rates *in vivo* (for reviews, see Rhoads, 1991; Frederickson and Sonenberg, 1993). It is thus possible that rapamycin prevents the phosphorylation of eIF-4E and consequently inhibits cap-dependent translation. We therefore examined the phosphorylation state of eIF-4E upon rapamycin treatment. eIF-4E phosphorylation was studied by isoelectric focusing (IEF) followed by immunoblotting. eIF-4E comprises two major isoelectric variants (pI 5.9 and 6.3) and the more acidic form is phosphorylated. In serum-starved NIH 3T3 cells, eIF-4E is underphosphorylated (15%) and addition of serum strongly induced phosphorylation of eIF-4E (75%) (Figure 6). Rapamycin, added in the presence of serum for 3 h, slightly reduced serum-induced phosphorylation of eIF-4E by ~12% (Figure 6). Thus, inhibition of translation by rapamycin in these experiments is not likely the result of dephosphorylation of eIF-4E.

Discussion

The results in this paper indicate that 4E-BP1 is a novel target for rapamycin. While the paper was under review, similar findings were reported by Graves *et al.* (1995) and Lin *et al.* (1995). This result appears *a priori* surprising in the light of earlier experiments indicating that 4E-BP1 is phosphorylated by MAP kinase (Haystead *et al.*, 1994; Lin *et al.*, 1994) and because rapamycin has no effect on the growth-stimulated activation of the MAP kinase/RSK signal transduction pathway (Chung *et al.*, 1992; Price *et al.*, 1992). However, recent results cast considerable doubt on the involvement of MAP kinases in phosphorylation of 4E-BP1 *in vivo*, because of the following: (i) PD 058059, an inhibitor of MEK, which phosphorylates MAP kinase, drastically inhibited insulin-stimulated MAP kinase activity, but had no effect on the phosphorylation of 4E-BP1 (Lin *et al.*, 1995); (ii) IGF-1 increased the phosphorylation of 4E-BP1 in aortic smooth muscle cells, without an increase in MAP kinase activity (Graves *et al.*, 1995); (iii) stimulation of 4E-BP1 phosphorylation by insulin occurred in the absence of MAP kinase activation in 293 cells (Von Manteuffel *et al.*, 1996) and (iv) wortmannin, which is a specific inhibitor of phosphatidylinositol 3′ kinase (PI3K), inhibited 4E-BP1 phosphorylation (Von Manteuffel *et al.*, 1996). Taken together, these findings and the results presented here strongly suggest that phosphorylation of 4E-BP1 is controlled by the TOR/FRAP signal transduction pathway.

Rapamycin blocks the phosphorylation of p70 S6 kinase in response to growth factors, hormones and other mitogens (Chung *et al.*, 1992; Price *et al.*, 1992; Thomas, 1993). However, 4E-BP1 is not a direct substrate for p70 S6 kinase and it does not contain the consensus phosphorylation site by p70 S6 kinase (Haystead *et al.*, 1994; A.-C.Gingras and N.Sonenberg, unpublished observations). It is thus possible that an upstream kinase in the p70 S6 kinase pathway phosphorylates 4E-BP1.

The findings described in this paper that rapamycin specifically inhibits cap-dependent translation in mamma-

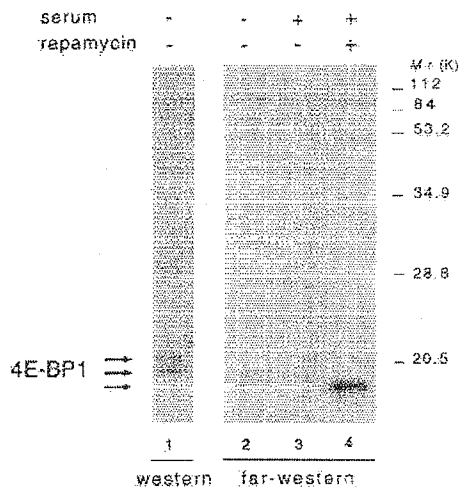

Fig. 5. Far-Western analysis of the interaction between eIF-4E and 4E-BP1. NIH 3T3 cells starved for serum for 48 h were treated with serum (10%) and rapamycin (20 ng/ml) for 3 h. Cells were lysed in 10 mM Tris–HCl, pH 7.5 buffer containing 5 mM EDTA and 2 mM EGTA. The homogenate was centrifuged at 6000 *g* for 10 min and the supernatant collected. Each sample (200 μg) was boiled for 7 min and the precipitated protein removed by centrifugation. The remaining protein was separated on an SDS–15% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with [^{32}P]labelled HMK–eIF-4E (2.5×10^5 c.p.m./ml) in 20 ml overnight at 4°C as described (Pause *et al.*, 1994) and autoradiographed (lanes 2–4). The binding of ^{32}P labelled HMK–eIF-4E was quantified using a phosphorimager. Lane 1 shows an immunoblot of an extract sample identical to that loaded on lane 2 probed with rabbit anti-4E-BP1 (1:1000).

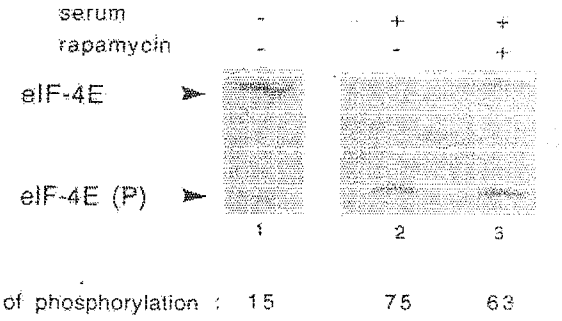

Fig. 6. Effect of rapamycin on eIF-4E phosphorylation in NIH 3T3 cells. NIH 3T3 cells serum-starved for 48 h were treated with 10% serum for 3 h in the presence or absence of 20 ng/ml rapamycin. Total protein extracts were fractionated by IEF PAGE and subjected to immunoblot analysis using polyclonal antibody to eIF-4E as described in Materials and methods. Arrows marked eIF-4E and eIF-4E (P) indicate the position of the non-phosphorylated and phosphorylated forms of eIF-4E, respectively. Phosphorylation of eIF-4E is expressed as the percentage of the phosphorylated form eIF-4E (P) in each sample. The experiment was carried out three times, with <5% variation between the experiments.

lian cells are in accord with recent observations obtained in yeast (Barbet *et al.*, 1996). In yeast, rapamycin is a potent inhibitor of translation and it was suggested that inhibition is cap-dependent (Barbet *et al.*, 1996). However, in contrast to mammalian cells, in yeast the effect on translation is totally independent of S6, as S6 phosphorylation is not required for translation and growth. It is striking, however, that the rapamycin-sensitive signalling pathway in yeast and mammals is very similar in the upstream components including the TOR/FRAP and FKBPs. It therefore appears that in yeast and mammals, rapamycin exerts its inhibition of translation by affecting an eIF-4E binding protein.

It was previously demonstrated that in several mammalian cell lines, rapamycin specifically represses the translation of mRNAs containing a polypyrimidine tract in the beginning of the 5′ untranslated region (Jefferies *et al.*, 1994; Terada *et al.*, 1994). This was shown to be due to the inhibition of the S6 phosphorylation pathway. Here we report a reduction in cap-dependent translation in response to rapamycin. However, it appears that the kinetics of inhibition of translation of polypyrimidine tract containing mRNAs occurs earlier than the general inhibition of capped mRNAs. As the dependence on the cap structure and eIF-4E for translation is directly correlated with the degree of secondary structure in the 5′ untranslated region (Pelletier and Sonenberg, 1985; Koromilas *et al.*, 1992), it is anticipated that translation of mRNAs containing extensive secondary structure would be more severely affected by rapamycin treatment than mRNAs with reduced secondary structure.

Inhibition of eIF-4E function by rapamycin is consistent with the effect of rapamycin on cell growth. eIF-4E is the rate-limiting factor in the cap-dependent initiation process and its overexpression in NIH 3T3 cells leads to transformation (Lazaris-Karatzas *et al.*, 1990). Conversely, inhibition of eIF-4E expression slows cell proliferation in mammalian cells (De Benedetti *et al.*, 1991) and results in an early G_1 arrest in yeast (Brenner *et al.*, 1988).

While 4E-BP1 is completely dephosphorylated following rapamycin treatment for ~1 h, cap-dependent translation is inhibited later and the inhibition is only partial. One explanation for the difference in kinetics is based on the finding that 4E-BP1 and p220 (p220 binds to eIF-4E in the cap binding complex, eIF-4F) directly compete for binding to eIF-4E (Haghighat *et al.*, 1995). The binding site of eIF-4E on p220 has been recently characterized (Lamphear *et al.*, 1995; Mader *et al.*, 1995). A similar binding site is present also in 4E-BP1 and mutations in the common sequence abrogate the binding of 4E-BP1 and p220 to eIF-4E (Mader *et al.*, 1995). eIF-4E exchanges slowly through the eIF-4F complex, and thus, would interact with dephosphorylated 4E-BP1 only after it is released from eIF-4F. Thus, translational inhibition would be dependent on the relative amounts of eIF-4E, 4E-BP1 and p220 and their relative binding affinities.

In summary, our results demonstrate that the inhibitory effect of rapamycin on cell growth can be explained at least partly as a consequence of the reduction of 4E-BP1 phosphorylation and eIF-4E function.

Materials and methods

Cells and antibodies

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Preceding rapamycin (Calbiochem) treatment, confluent plates were serum-starved for 48 h.

Antibody 11208 was raised in rabbit (Pocono farm) against a GST–

4E-BP1 fusion protein. It does not cross-react with 4E-BP2, a protein which is 56% identical to 4E-BP1. The antibody recognizes both human and mouse 4E-BP1.

Metabolic labelling

NIH 3T3 cells were serum-starved for 48 h and pre-incubated for 1 h in methionine-free medium. [^{35}S]Methionine (100 μCi) and 10% FCS were added. Cells were lysed in buffer containing 0.5% NP-40, 140 mM NaCl and 30 mM Tris–HCl, pH 7.5, and radioactivity incorporated into TCA precipitable material was measured.

In vivo [^{32}P]orthophosphate labelling and immunoprecipitation

NIH 3T3 cells were serum-starved for 48 h, rinsed twice in phosphate-free DMEM (Gibco) and [^{32}P]orthophosphate (0.5 mCi/ml; NEN-Dupont) was added and cells were incubated for 1 h. Treatment of cells with serum and rapamycin was performed for 3 h. The medium was removed and cells were rinsed twice in cold PBS and lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Incorporation of radioactivity into TCA-precipitated material was determined and samples containing 500 000 c.p.m. were incubated for 2 h at 4°C with anti 4E-BP1 antibody preadsorbed on protein A–agarose beads. Beads were washed three times in RIPA buffer and resuspended in Laemmli sample buffer (Laemmli, 1970).

Transfections and extract preparation

NIH 3T3 cells were infected for 1 h in serum-free medium with the recombinant vaccinia virus vTF7-3 (Fuerst *et al.*, 1986). Cells were transiently transfected with the plasmid pGEMCAT/EMC/LUC (2 μg) or the plasmid pGEMLUC/pol/CAT (2 μg) using lipofectin (15 μl) as previously described (Pause *et al.*, 1994). Cell extracts were prepared 20 h post-transfection and analysed for CAT expression by enzyme immunoassay (Boehringer) and LUC activity was measured in millivolts in a luminometer (BIOORBIT) using a LUC assay system (Promega).

Western blotting

Cells were lysed by successive freeze–thaw cycles, in 20 mM Tris–HCl, pH 7.5 buffer containing 5 mM EDTA, 2 mM EGTA and 100 mM KCl. The homogenate was centrifuged at 6000 *g* for 10 min and the supernatant was collected. To analyse for 4E-BP1, 50 μg of protein were dissolved in Laemmli sample buffer (Laemmli, 1970) and the samples were loaded onto an SDS–15% polyacrylamide gel. Proteins were transferred onto a 0.22 μm nitrocellulose membrane, which was blocked in 5% milk for 2 h followed by incubation for 2 h with rabbit polyclonal antiserum against 4E-BP1 (1:1000) in 10 mM Tris–HCl, pH 8.0 buffer containing 150 mM NaCl. Incubation with [^{125}I]protein A (Amersham) was performed (1:1000) and the signal was quantified using phosphorimager analysis.

Far-Western analysis

A fusion protein containing the HMK phosphorylation site and eIF-4E (HMK-eIF-4E) was expressed in *Escherichia coli* BL 21 cells and purified using a m^{7}GDP affinity column as described (Pause *et al.*, 1994). HMK–eIF-4E was phosphorylated using [γ-^{32}P]ATP and bovine HMK (Sigma).

Heat-treated extracts were processed on an SDS–15% polyacrylamide gel and proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk in HBB (25 mM HEPES–KOH, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40) for 2 h. Membranes were then incubated for 12 h at 4°C in hybridization buffer (20 mM HEPES–KOH, pH 7.7, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, 1% skim milk) containing ^{32}P-labelled HMK–eIF-4E probe at 250 000 c.p.m./ml, as described (Pause *et al.*, 1994). After three washes with hybridization buffer, the membranes were dried and exposed against an X-ray film.

Analysis of eIF-4E phosphorylation by IEF

Protein samples from NIH 3T3 cells were subjected to IEF essentially as described (Maurides *et al.*, 1989). Cells were lysed in IEF sample buffer and subjected to IEF using a pH range of 5–7, in the presence of 9 M urea, 50 mM dithiothreitol and 2% CHAPS (Fluka). Proteins were electrophoresed at 5 mA/gel for 16 h, with 0.01 M glutamic acid at the cathode and 0.02 M NaOH at the anode. Proteins were transferred onto a 0.22 μm nitrocellulose membrane which was blocked in 5% milk for 2 h followed by incubation for 2 h with rabbit polyclonal antiserum against eIF-4E (1:3000) in 10 mM Tris–HCl, pH 8.0 buffer containing 150 mM NaCl. Incubation with [^{125}I]protein A (Amersham) was performed (1:1000) and the signal was quantified using a PhosphorImager.

In vitro translation

Plasmid pGEMCAT/EMC/LUC was *in vitro* transcribed with T7 RNA polymerase (Promega). Following incubation, the mRNAs were phenol–chloroform extracted, purified by gel filtration on nick-columns (Pharmacia) and ethanol-precipitated. mRNA integrity was checked by formaldehyde–agarose gel electrophoresis and ethidium bromide staining.

Mouse Krebs-2 ascites carcinoma cells were grown in Balb/c mice for 7 days. S_{30} extracts were prepared and treated with micrococcal nuclease (Svitkin and Agol, 1978). Translation was performed with [^{35}S]methionine in a volume of 12.5 μl at 30°C for 90 min, as described (Svitkin *et al.*, 1984), in the presence of 0.15 μg mRNA.

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Zotarolimus (ABT-578) eluting stents ☆

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Abstract

Drug-eluting stents have revolutionized the field of interventional cardiology and have provided a significant innovation for preventing coronary artery restenosis. Polymer coatings that deliver anti-proliferative drugs to the vessel wall are key components of these revolutionary medical devices. This article focuses on the development of stents which elute the potent anti-proliferative agent, zotarolimus, from a synthetic phosphorylcholine-based polymer known for its biocompatible profile. Zotarolimus is the first drug developed specifically for local delivery from stents for the prevention of restenosis and has been tested extensively to support this indication. Clinical experience with the PC polymer is also extensive, since more than 120,000 patients have been implanted to date with stents containing this non-thrombogenic coating. This review provides background on pre-clinical studies with zotarolimus, on the development of the biocompatible PC polymer and on the clinical trials conducted using two stent platforms which deliver this drug to patients with coronary artery disease.

Keywords: Restenosis; Zotarolimus; Coronary stenting; Coronary artery disease

Contents

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1. Introduction

When Andreas Gruentzig completed the first coronary artery dilatation in a canine model nearly 30 years ago, occlusive coronary artery disease had long been a challenge to interventional cardiologists, addressed only by bypass surgery. Gruentzig's innovative study led to a more significant event in 1977, when the first patient was successfully dilated in Zurich, marking the first percutaneous coronary intervention (PCI) conducted using a balloon catheter in man [1]. Interventional cardiologists rapidly accepted PCI, a procedure designed to restore normal blood flow in vessels narrowed by vascular disease. Despite the initial benefits derived from balloon angioplasty, it soon became evident that renewed obstruction of the vasculature accompanied these interventions in as many as 30–50% of patients, depending on the extent of the underlying disease [2–5]. This effect, termed restenosis, remained a serious problem in many patients until the introduction of intracoronary stents in the late 1980s. These metal scaffolds, used to enlarge and maintain the lumenal diameter of the treated vessel and to prevent recoil, significantly reduced the restenosis rate associated with balloon angioplasty. Still, the successful outcome was often limited in duration by subsequent restenosis of the vessel, an event typically referred to as in-stent restenosis. This response to mechanical injury and to the presence of a metal scaffold is mainly characterized by proliferation of vascular smooth muscle cells [6]. In-stent restenosis re-occluded the vessel in more than 30% of the patients who received these bare metal devices [7–11]. A more recent approach, designed to prevent in-stent restenosis, has been the use of drug-eluting stents coated with polymers to deliver anti-proliferative agents such as rapamycin [12,13] or paclitaxel [14,15]. Recent introduction and widespread use of these coated devices has now reduced the restenosis rate to less than 10%.

At least three major elements must be considered when designing a drug-eluting stent system for optimal performance in patients with coronary artery disease. First, the drug should robustly inhibit the proliferation of coronary vascular smooth muscle cells, the target cell population. It should also possess certain sanguine chemical properties, such as stability and lipophilicity, which enhance and sustain delivery to the lipid-rich arterial wall. Second, the polymer that covers the stent struts and contains the drug must be durable, displaying physical characteristics, which allow the incorporation and release of the appropriate drug and be composed of a biocompatible material that minimizes the possibility of hypersensitivity reactions and thrombus formation. Finally, the stent itself must possess appropriate operating characteristics, including symmetric scaffolding of the arterial surface for uniform drug delivery, radiopacity, flexibility and ease of delivery to the lesion. Particularly useful are sufficiently thin stent struts, which have been conclusively shown to generate less injury and neointimal hyperplasia [16–19]. Each of these key elements will be further described in the sections to follow.

2. Zotarolimus

The inherent growth inhibitory properties of many anti-cancer agents make these drugs ideal candidates for the prevention of restenosis. However, these same properties are often associated with cytotoxicity at doses which block cell proliferation. Therefore, the unique cytostatic nature of the immunosuppressant rapamycin was the basis for the use of this drug on the Cypher™ stent recently developed by Johnson and Johnson. Rapamycin was originally approved for the prevention of renal transplant rejection in 1999 [20]. More recently, Abbott Laboratories developed a compound from the same class, zotarolimus (formerly ABT-578), as the first cytostatic agent to be used

solely for delivery from drug-eluting stents to prevent restenosis [21,22]. A large body of pre-clinical evidence was compiled to support this approach and will be described in the next section.

Zotarolimus is a proprietary analog made by substituting a tetrazole ring for the native hydroxyl group at position 42 in rapamycin that is isolated and purified as a natural product from fermentation. This site of modification was found to be the most tolerant position to introduce novel structural changes without impairing biologic activity. The compound is extremely lipophilic, with a very high octanol:water partition coefficient, and therefore has limited water solubility. These properties are highly advantageous for designing a drug-loaded stent containing zotarolimus in order to obtain a slow sustained release of drug from the stent directly into the wall of coronary vessels. The poor water solubility prevents rapid release into the circulation, since elution of drug from the stent will be partly dissolution rate-limited. The slow rate of release and subsequent diffusion of the molecule facilitates the maintenance of therapeutic drug levels eluting from the stent. In addition, its lipophilic character favors crossing cell membranes to inhibit neointimal proliferation of target tissue. The octanol:water partition coefficients of a number of compounds, recently obtained in a comparative study, indicate that zotarolimus is the most lipophilic of all DES drugs (Fig. 1, [23]).

The key biologic event associated with the restenotic process is clearly the proliferation of smooth muscle cells in response to the expansion of a foreign body against the vessel wall [24–27]. This proliferative response is initiated by the early expression of growth factors such as PDGF isoforms [28], bFGF [29], and thrombin [30], which bind to cellular receptors. However, the key to understanding the mechanism by which compounds like zotarolimus inhibit cell proliferation is based on events which occur downstream of this growth factor binding. The signal transduction events which culminate in cell cycle arrest in the G1 phase are initiated as a result of ligand binding to an immunophilin known as FK binding protein-12 [31]. The FK designation was based on early studies conducted with tacrolimus, formerly known as FK-506, which binds this cytoplasmic protein with high affinity [31]. Subsequent investigations showed that rapamycin also binds to this intracellular target, forming an FKBP12–rapamycin complex which is not in itself inhibitory, but does have the capacity to block an integral protein kinase known as target of rapamycin (TOR) [32,33]. TOR was first discovered in yeast [33,34] and later identified in eukaryotic cells, where it was designated as mTOR, the mammalian target of rapamycin. The importance of mTOR is based on its ability to phosphorylate a number of key proteins, including those associated with protein synthesis ($p70^{s6}$kinase

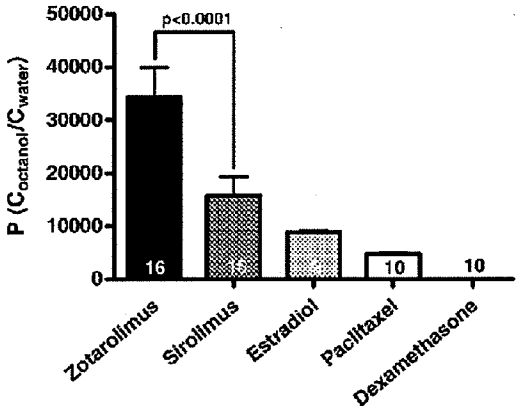

Fig. 1. Octanol/Water partition coefficients for five compounds, which have been delivered from drug-eluting stents. Zotarolimus is the most lipophilic of all DES drugs tested.

[35]) and initiation of translation (4E-BP1) [36–38]. Of particular significance is the role that mTOR plays in the regulation of $p27^{kip1}$, an inhibitor of cyclin-dependent kinases such as cdk2. The binding of agents like rapamycin and zotarolimus to mTOR is thought to block mTOR's crucial role in these cellular events, resulting in arrest of the cell cycle, and ultimately, cell proliferation.

To characterize the mode of action of zotarolimus relative to rapamycin, the affinity of the compounds for binding to human recombinant FKBP-12 was determined in a competitive binding assay. Competition for binding was measured using an analog of ascomycin, which competes with FK506, as well as sirolimus family members, for binding to the FKBP-12 immunophilin protein (Fig. 2). The compounds were tested in a dose–response fashion in 11 separate experiments, resulting in an IC_{50} of 2.8 ± 0.39nM (mean ± SEM) for inhibition of FKBP-12 binding by zotarolimus. The FKBP-12 binding affinity of zotarolimus was slightly lower than that for rapamycin (1.7 ± 0.16nM), but within a range that would be expected to result in similar biologic effects. The results demonstrate that zotarolimus retains the critical structural features required to interact with FKBP-12, a requisite for inhibiting mTOR-dependent growth factor stimulation. The tight binding of zotarolimus to FKBP-12 is consistent mechanistically with its potent inhibitory activity on cell proliferation.

Additional studies showed that this effect translates into inhibition of leukocyte proliferation, as evidenced by blockade of the mixed lymphocyte reaction in a variety of species, including rat, mouse, swine and

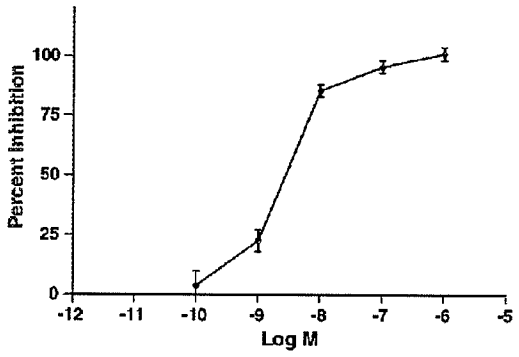

Fig. 2. Inhibition of ascomycin binding to FKBP-12 by zotarolimus. Symbols represent mean ± SEM; $n = 11$.

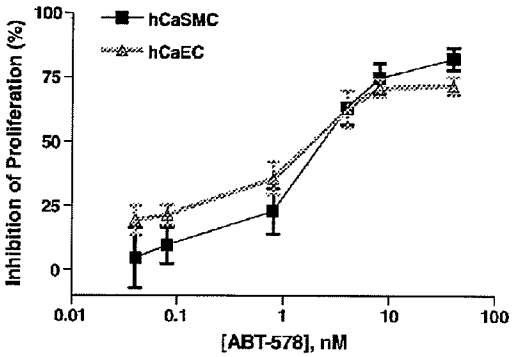

Fig. 3. Inhibition of cultured human coronary artery smooth muscle cells (hCaSMC) and human coronary artery endothelial cells (hCaEC) by zotarolimus. IC_{50}s are 2.9 ± 0.3nM and 2.6 ± 0.4nM for hCaSMC and hCaEC, respectively. Symbols represent mean ± SEM; $n = 6$.

human. However, the most relevant in vitro data to support a restenosis indication are that which show the clear inhibition of human coronary artery smooth muscle cell proliferation. To study the ability of the drug to inhibit proliferation of this cell type, incorporation of ^{3}H-thymidine into newly synthesized DNA of cells stimulated by serum and growth factors was studied in the presence and absence of zotarolimus. The results of these studies are shown in Fig. 3. Zotarolimus inhibits proliferation of human smooth muscle cells with an IC_{50} in the low nanomolar range (2.9 ± 0.7nM, mean ± SEM). Similar results have been shown in canine smooth muscle cells [39]. Endothelial cell proliferation was also inhibited ($IC_{50} = 2.6 \pm 1.0$nM). However, despite these in vitro results, data from animal studies indicate near-complete restoration of endothelial cell growth at 30 days after implant of zotarolimus-eluting stents.

A strong mechanistic rationale based on inhibition of FKBP-12 binding and inhibition of cell proliferation, combined with clear demonstration of appropriate physical–chemical characteristics, prompted the completion of a definitive study in a classic swine model to determine the safety and efficacy of zotarolimus. Twenty phosphorylcholine-coated (PC) stents, and 20 PC stents eluting zotarolimus were placed in 20 domestic crossbred juvenile swine. After 28 days, coronary arteries that were implanted with zotarolimus stents developed 50% less stenosis, 50% less neointimal area, 42% less neointimal thickness

and 35% greater lumen area relative to PC stents without drug. All arteries in both the polymer only and polymer/drug stent showed near-complete healing and minimal toxicity. It was concluded that, in 28-day porcine coronary arteries, the release of zotarolimus from PC coated stents is feasible, safe and effective for in-stent neointimal reduction in the porcine model. These results clearly suggested safety and efficacy, with promising potential for human coronary stenting [40].

3. Zotarolimus human safety studies

Before initiating human studies to deliver zotarolimus from coronary stents, a series of single- and multiple-dose evaluations were conducted to determine the safety and pharmacokinetic profile in healthy subjects. The single-dose study was a Phase I, escalating double-blind, randomized placebo-controlled study conducted in 60 healthy adult males, divided into groups of 12 subjects each. These volunteers received intravenous doses of 100, 300, 500, 700 and 900μg zotarolimus or matching placebo. Blood samples were obtained for 168 h to determine blood concentrations using liquid chromatography/mass spectrometry methods [41]. The higher doses were not administered until safety data from preceding lower doses had been evaluated. The safety evaluations were based on determination of adverse events, physical examinations, vital signs, ECG, examination of the injection site and assessment of laboratory tests. The results revealed dose-proportional increases in C_5 (blood concentration at 5 min), $AUC_{0\text{-last}}$, and $AUC_{0\text{-inf}}$, indicating linear pharmacokinetics of zotarolimus across the dose range tested. Of particular importance was the finding that these doses were generally well-tolerated by the subjects, that no serious adverse events were reported and that no clinically significant changes in the physical examinations, vital signs, clinical laboratory or ECG parameters were observed [42].

A subsequent study was then conducted to determine the pharmacokinetic profile of 14 consecutive intravenous doses of zotarolimus in 72 healthy subjects. In this Phase I double-blind, placebo-controlled randomized study, the patients were equally divided to receive 3 once daily regimens of 200, 400 or 800μg of zotarolimus (16 subjects) or placebo (8 subjects) for each regimen. Dosing occurred for 14 consecutive days, followed by collection of blood and urine samples at selected time points. Steady state for once daily dosing of zotarolimus was reached by Day 10. Evaluation of the urine samples using a combination of HPLC and MS/MS techniques [43] indicated that renal excretion is not a major route of elimination for zotarolimus, since only 0.1% of the dose was excreted unchanged in the urine each day. As indicated in the single-dose studies described earlier, there were no serious adverse events associated with zotarolimus dosing when administered at these dose levels. None of the subjects displayed evidence of immunosuppression, QTc prolongation or other clinically significant adverse events. Completion of these studies cleared the way for the start of clinical trials to evaluate stent drug delivery of zotarolimus.

4. Phosphorylcholine polymer

For targeted local delivery of zotarolimus to the artery, the drug is incorporated into a methacrylate-based copolymer that includes a synthetic form of phosphorylcholine, the zwitterionic phospholipid found in the outer surface of red blood cell membranes. This use of biomimicry, or the practice of using polymers that occur naturally in biology, provides a coating, now described as PC, with minimal thrombus deposition [44] and no adverse clinical effect on late healing of the vessel wall. The first application of this approach for use on stents evolved from efforts by Hayward and Chapman [45], who demonstrated that the phosphorylcholine (PC) component of the outer surface of the erythrocyte bilayer was non-thrombogenic. To date, more than 120,000 PC-coated stents have been implanted in patients with no apparent deleterious effect in the long term compared to bare metal stent technologies. Not only is the coating non-thrombogenic, but it also exhibits other features that should be present when applying such a material to a medical device for long-term implantation. These include durability, neutrality to the chemistry of the incorporated drug and an ability for sterilization using standard methods which do not affect drug structure or efficacy [46].

5. Clinical trials

5.1. The PREFER zotarolimus-eluting stent

The first clinical trial of a zotarolimus-eluting coronary stent was the PREFER trial, sponsored by Abbott Laboratories, using the PC coated Bio*divYsio*® stent which was acquired from Biocompatibles, Ltd. in 2002. The PREFER IVUS pilot trial was "A Prospective study to evaluate the safety and efficacy of the ABT-578 coated Bio*divYsio* stent for the reduction of restenosis", and was designed to evaluate the primary endpoint of 30-day Major Adverse Cardiac Event rate (MACE) in 50 patients. Secondary endpoints included target vessel revascularization, as well as late term MACE, evaluated at 6 months, 1 and 5 years. The enrolled patients had 3.0 mm diameter single de novo coronary lesions of less than 15 mm in length, and received Zotarolimus PC-coated Bio*divYsio* stents (3.0 mm diameter × 18 mm in length). Stents were deployed in all 11 patients with 100% lesion and device success and no stent thromboses were observed. While one patient experienced an in-hospital MACE event related to side-branch occlusion, no significant in-hospital clinical events were observed in the remaining ten subjects. An internal Abbott decision to limit this evaluation to an initial 11 patients was made so that the pre-clinical animal studies could be expanded, and so that the stent platform could be changed to the Triplex stent (see below). However, a full IVUS and angiographic evaluation of the 11 patients who were implanted with the Bio*divYsio* Zotarolimus-eluting stent system was completed at 90 days. At this time point, mean acute gain was 1.6 ± 0.4 mm, late loss was 0.1 ± 0.3 mm and late loss index (the loss/gain ratio) was 0.1 ± 0.2 mm. These subjects have now been followed to 360 days. MACE was observed in only 1 of 11 patients, indicating support for the safety of zotarolimus in this population.

5.2. The ENDEAVOR zotarolimus-eluting stent

Abbott Laboratories licensed zotarolimus and PC for use by Medtronic Vascular on the Endeavor Stent. The Endeavor stent uses the cobalt-chromium Driver™ stent as a platform for drug elution [47].

The ENDEAVOR I clinical study was conducted by Medtronic Inc. to evaluate the safety and efficacy of zotarolimus on the Endeavor stent for the treatment of single de novo lesions in native coronary arteries [21]. The Endeavor stent was deployed in 100 patients (93 treated with a single zotarolimus coated stent, and 7 who received a second Endeavor stent). The study was a prospective, single-arm, multicenter trial initiated in 6 Australian sites and 2 study sites in New Zealand. The primary endpoints were MACE at 30 days and angiographic late loss at 4 months. Secondary endpoints included IVUS and angiographic assessments at 4 and 12 months, as well as clinical follow-up at 9 and 12 months, and annually for 5 years.

The coronary artery lesions in the enrolled patients were ≤15 mm in length and ≥3.0 mm to ≤3.5 mm in diameter. The acute clinical results demonstrated 100.0% (100/100) lesion, device and procedure success rates with hierarchical MACE rate of 1.0% (1/100) at 30 days. Four-month angiographic late loss was 0.21 ± 0.40 in-segment and 0.33 ± 0.36 in-stent and neointimal hyperplastic volume by IVUS was 6.1 mm^3. Twelve-month angiographic late loss was 0.43 ± 0.44 in-segment and 0.61 ± 0.44 in-stent for the 93 evaluable patients and neointimal hyperplastic volume by IVUS was 14.2 mm^3. The cumulative hierarchical incidences of TLR, TVF and MACE at 12 months were all 2.0% (2/100).

5.3. The ENDEAVOR II trial

This multicenter trial (Randomized controlled trial to evaluate the safety and efficacy of the Medtronic AVE zotarolimus-eluting Driver coronary stent in de novo native coronary artery lesions) compared the safety and efficacy of the Endeavor stent system with the uncoated Driver™ coronary stent. The study was conducted in 1200 patients (600 in each treatment arm) who presented with coronary lesions in vessels of 2.25 3.5 mm in diameter and 14 27 mm in length. The primary endpoint was 9-month TVF rate, defined as a composite of target vessel revascularization, recurrent Q or non Q-wave MI, or cardiac death that could not be clearly attributed to a vessel other than the target vessel. The MACE rate at 30 days was the primary safety endpoint. Among the secondary endpoints were 8-month late loss as measured by QCA (N=300 per group), in-stent and in-lesion binary restenosis at 8 months, neointimal hyperplastic

volume at 8 months (as measured by IVUS), and 9-month assessments of TLR and TVR.

The statistical analysis for the primary endpoint assumed a 9-month TVF rate of 16% in the control arm and 9.5% in the Endeavor arm of the study. This implies a treatment effect of approximately 40%, requiring a sample size of 552 patients per treatment arm (1104 in total) to provide 90% power to achieve this effect. A total of 1200 patients were enrolled to account for a loss to follow-up of 8%. Preliminary results indicate that the primary endpoint was achieved in this study, with a significant reduction from 15.4% for the bare metal arm to 8.1% ($P<0.0005$) for the Endeavor stent. This reduction was similar to that achieved in the Cypher™ SIRIUS trial (21% to 8.6%) and in the Taxus IV trial (14% to 7.6%). Further comparisons of the Endeavor II results with those from these pivotal trials indicate similar MACE rates of 7.4%, 7.1% and 7.6% for Endeavor II, Cypher™ and Taxus™ trials, respectively. In-stent late lumen loss generated by the Endeavor stent (0.62 ± 0.46 mm) was similar to Endeavor I (0.61 ± 0.44 mm) and higher than Cypher (0.17 ± 0.45) or Taxus (0.39 ± 0.50), but this finding had no impact on the clinical results.

5.4. The ENDEAVOR III trial

This randomized, single-blind trial ("A randomized controlled trial of the Medtronic Endeavor™ coronary stent versus the Cordis Cypher™ sirolimus-eluting coronary stent system in de novo native coronary artery lesions") was conducted in 480 patients who presented with native coronary lesions ranging from 10 to 27 mm in vessels of 2.5–3.5 mm in diameter. The study is designed to evaluate non-inferiority of the Endeavor™ stent ($n=240$) relative to the currently marketed Cypher™ stent ($n=240$) for the reduction of restenosis. The primary endpoint is in-stent late lumen loss at 8 months, as measure by QCA, where in-stent late lumen loss is defined as the difference between the post-procedure minimal lumen diameter (MLD) and the follow-up angiography MLD. As indicated in the studies described earlier, the primary safety endpoint is 30-day MACE. The secondary endpoints include in-stent and in-lesion MLD at 8 months post-procedure, TLR, TVR and TVF rates and 8-month neointimal hyperplastic volume as measured by IVUS.

The trial was designed with 90% statistical power to detect an in-segment late loss of ≤0.20 mm, relative to the anticipated late loss of 0.24 mm in the Cypher™ arm of the study. The study is fully enrolled, and the results are expected to be reported in 2005.

5.5. The ZoMaxx zotarolimus-eluting stent

The third zotarolimus-eluting coronary stent to reach clinical trials is the ZoMaxx™ stent. Developed by Abbott Vascular, the ZoMaxx™ stent was designed for thin strut width, low profile, and high radial strength, while still maintaining adequate visibility on fluoroscopy. The stent is made of Triplex™ metal, a trilayer composite with two outer layers of 3161 stainless steel and an inner layer of tantalum (Fig. 4) [48]. The inclusion of this 0.0007″ tantalum layer provides radiopacity, while the stainless steel components provide sufficient hoop strength to minimize vessel recoil. Coronary stents made of tantalum metal have been used in patients with good procedural success, based in part on the ease of visualization associated with the use of this material [49–51]. Clinical experience which documents the safety of tantalum-containing stents has been widely published [50,52–55].

It has been suggested that stents with thin struts induce minimal neointimal proliferation [16–19]. This is a key characteristic of the ZoMaxx™ stent, which features a strut thickness of only 0.0029″ [48]. In addition, the thin struts generate an extremely low

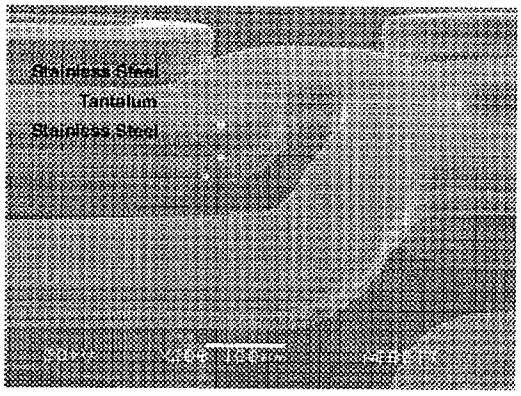

Fig. 4. The Triplex™ Stent, a stainless steel/tantalum/stainless steel composite.

crossing profile, so that the outer diameter of the 3.0 mm stent crimped onto the balloon is less than one millimeter. Furthermore, the novel ZoMaxx™ pattern includes two connectors between the rings of the stent to confer optimum flexibility, allowing improved delivery to vessels, which are often tortuous and irregular. Finally, the inclusion of 8–10 cells around the stent perimeter sub-divides the supported vessel area and ultimately provides for optimum scaffolding. The incorporation of more than the typical 6 cells into the ZoMaxx™ design provides a stent with less distance between struts, and theoretically, a more even distribution of drug to the vessel wall.

The ZoMaxx™ stent delivers its antiproliferative agent through a series of PC polymer coats, so that the completed stent displays experimental elution kinetics qualitatively similar to the Cypher™ stent [56]. A series of in vitro and in vivo studies were conducted to establish an elution profile, which would deliver drug over a time frame consistent with events associated with the restenotic process. It was determined that drug elution could be controlled by placement of a PC topcoat over a PC/drug layer, and that the elution kinetics were dependent on the thickness of this top layer [57]. Results of these studies indicated that the most rapid drug elution occurred when no topcoat was present, and that elution was slowed in a graded fashion when topcoats of increasing thickness were applied. Therefore, during the first week after implant, a PC topcoat of 5 μg/mm of stent length resulted in a drug release of approximately 60% during the first week after implant, followed by an additional 20% during the second week. The remaining 20% is released over the next 2 weeks, so that virtually all drug has been eluted over a 1-month period [56].

5.6. The ZoMaxx™ I clinical trial

ZoMaxx I is a randomized, controlled trial designed to evaluate the safety and efficacy of the ZoMaxx Drug-Eluting Coronary Stent System compared to the TAXUS™ Express²™ Paclitaxel-Eluting Coronary Stent System in de novo coronary artery lesions. Its principal investigator is Dr. Bernard Chevalier. The study is being conducted in 400 patients in Europe, Australia and New Zealand with ischemic heart disease due to stenotic lesions of native coronary arteries with reference vessel diameter ≥2.5 mm and ≤3.5 mm and lesion lengths ≥10 mm and ≤30 mm. The primary endpoint is in-segment late lumen loss at 9 months (as measured by QCA), defined as the difference between the post-procedure minimal lumen diameter (MLD) and the follow-up angiography MLD. Secondary endpoints include in-stent late lumen loss, in-stent and in-lesion MLD and binary restenosis, and MACE, TLR, TVR and TVF rates at 9 months post-procedure. A cohort of 250 patients will be further evaluated with intravascular ultrasound (IVUS).

The trial was designed to demonstrate non-inferiority of in-segment late loss after nine months (expected difference in means, μZoMaxx Stent–μTaxus Stent=0), with a non-inferiority margin of 0.25 mm and standard deviation of 0.4 mm. The trial is 99% powered to detect non-inferiority with a one-sided α of 0.05. Enrollment in the ZoMaxx I trial was initiated in September 2004 and completed in July 2005.

5.7. The ZoMaxx™ II clinical trial

Similarly, ZoMaxx II is a randomized, controlled trial designed to evaluate the safety and efficacy of the ZoMaxx Drug-Eluting Coronary Stent System compared to the TAXUS™ Express²™ Paclitaxel-Eluting Coronary Stent System in de novo coronary artery lesions. This pivotal study seeks to evaluate a larger cohort of patients using a clinical endpoint (TVR at 9 months post-procedure). Its principal investigators are Drs. Alan C. Yeung and William Gray. The study is being conducted in 1670 patients with ischemic heart disease due to stenotic lesions of native coronary arteries with reference vessel diameters ≥2.5 mm and ≤3.75 mm and lesion lengths ≥10 mm and ≤24 mm that can be covered by one 2.5 mm diameter stent, or lesion lengths ≥10 mm and ≤28 mm that can be covered by one 3.0 or 3.5 mm diameter stent. The primary endpoint is ischemia-driven TVR at 9 months post-procedure. Secondary endpoints include in-stent and in-segment late lumen loss, MLD and binary restenosis, and MACE TLR, and TVF rates at 9 months post-procedure.

The trial was designed to demonstrate non-inferiority of TVR after 9 months (expected difference in means, μZoMaxx Stent–μTaxus Stent=0), with a non-inferiority margin of 3.6%. The trial is over 95%

powered to detect non-inferiority with a one-sided α of 0.05. Enrollment in the ZoMaxx II pivotal trial was initiated in the United States in May 2005.

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